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PRIMER NOTE

DEVELOPMENT OF THE FIRST CHLOROPLAST MICROSATELLITE LOCI IN *GINKGO BILOBA* (GINKGOACEAE)[1]

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- *Premise of the study:* To investigate population genetics, phylogeography, and cultivar origin of *Ginkgo biloba*, chloroplast microsatellite primers were developed.
- *Methods and Results:* Twenty-one chloroplast microsatellite markers were identified referring to the two published chloroplast genomes of *G. biloba*. Polymorphisms were assessed on four natural populations from the two refugia in China. Eight loci were detected to be polymorphic in these populations. The number of alleles per locus ranged from three to seven, and the unbiased haploid diversity per locus varied from 0.441 to 0.807.
- *Conclusions:* For the first time, we developed 21 chloroplast microsatellite markers for *G. biloba*, including 13 monomorphic and eight polymorphic ones within the assessed natural populations. These markers should provide a powerful tool for the study of genetic variation of both natural and cultivated populations of *G. biloba*, as well as cultivars.

Key words: chloroplast microsatellite; cpSSR; genetic diversity; *Ginkgo biloba*; Ginkgoaceae; gymnosperm; molecular marker.

Ginkgo biloba L., a "living fossil" and one of the most mysterious plant species, is the only extant representative of the isolated gymnosperm order Ginkgoales (Zhou, 2009). It became extinct in North America and Europe, and only survived the recurrent glaciation as a relic in China (Kwant, 2013). Recent molecular and ecological evidence strongly supports the existence of two refugia in China (Gong et al., 2008a, 2008b; Tang et al., 2012). Molecular data also confirmed an out-of-China distribution history mediated by anthropogenic introduction (Zhao et al., 2010). This fascinating plant has been cultivated worldwide for its medicinal and nutritional uses, its power as a source of artistic and religious inspiration, and its importance as one of the world's most popular street trees. Thus, it is imperative to increase our knowledge of its evolutionary history to support our efforts to conserve the refugial (natural) populations. Chloroplast DNA (cpDNA) sequences are prevalent markers used in population genetic studies. However, the relatively low resolution of cpDNA sequences of *Ginkgo* L. did not fully satisfy the inference of its evolutionary processes (Gong et al., 2008a, 2008b). Here, we report a set of polymorphic and monomorphic chloroplast microsatellite or simple sequence repeat (cpSSR) markers for *G. biloba*. They are expected to be particularly valuable, together with the published nuclear microsatellite markers (Yan et al., 2006, 2009), not only in inferring the evolutionary history but also for breeding of *G. biloba*.

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METHODS AND RESULTS

Two complete chloroplast genomes of *G. biloba* (NC_016986 and AB684440) were downloaded from GenBank and manually aligned using Geneious version 4.8.5 (Drummond et al., 2008). Polymorphic mononucleotide repeats longer than nine nucleotides were spotted directly from the alignment. Di-, tri-, tetra-, and pentanucleotide motifs with a minimum of six repeats were identified from one of the chloroplast genomes using SSRHunter version 1.3 (Li and Wan, 2005). A total of 27 chloroplast simple sequence repeat loci (cpSSRs) were identified, including 22 mononucleotide repeats and five dinucleotide repeats. Primers for these 27 cpSSRs were designed using Primer Premier version 5.0 (Clarke and Gorley, 2001) following the criteria: (1) GC content 40–60%; (2) melting temperature (T_m) 50–60°C; (3) primer size 18–22 bp in length; and (4) amplicon size 100–400 bp in length.

Primer pairs were initially screened for amplification success using four individuals of *G. biloba* from a Tianmu Mountain population. Genomic DNA was extracted from silica gel–dried leaf materials using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). PCR was performed in a 15-μL reaction mixture containing 60–90 ng genomic DNA, 0.75 U *Taq* polymerase (TaKaRa Biotechnology Co., Dalian, China), 0.8 μL 10× PCR buffer ($MgCl_2$ free), 2 mM $MgCl_2$, 0.12 mM dNTPs, and 0.33 μM of each primer, 6.67 mM bovine serum albumin (BSA, TaKaRa Biotechnology Co.). The amplification programs were as follows: initial denaturation at 94°C for 5 min; 30 cycles of 30 s at 94°C, 30 s at the optimized annealing temperature (Table 1), and extension for 40 s at 72°C; and a final extension step at 72°C for 10 min. Amplification products, along with a DL2000 DNA ladder (TaKaRa Biotechnology Co.), were electrophoresed on 2.0% agarose gels stained with ethidium bromide to assess successful amplification. Twenty-one of the 27 primer pairs

TABLE 1. Characteristics of 21 chloroplast microsatellite primers developed in *Ginkgo biloba*.

Locus[a]	Gene[b]	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	Size range (bp)	GenBank accession no.
GCP5 (HEX)	*petN-psbM**	$(T)_{11}$	F: ATATGACGAACCCCTCCCGC R: TTCTCCTGTGACAAATCCGA	55	171–175	KC344393
GCP6 (TAMRA)	*trnC*-GCA-*petN**	$(T)_{10}$	F: CCACTTCTTCCCCATACTAC R: GAATCTCTCAGGTTGACTCG	55	226–229	KC344394
GCP22 (FAM)	*psbK-psbL**	$(G)_8(A)_{12}$	F: TCCCCATTTATCCGTTTGTT R: CGGGATCAGTATTGGAAGTG	55	245–250	KC344388
GCP23 (HEX)	*trnG*-GCC*	$(A)_{12}$	F: GAAATAGGGAACGAAGTAAC R: GTGTAAAGGGGTATGAAGAA	55	311–320	KC344389
GCP24 (TAMRA)	*atpF-atpH**	$(G)_{13}$	F: GAACGGACTAAGTATGGAAA R: CAGAGGGTAAAATACGAGGT	55	260–262	KC344390
GCP20 (FAM)	*chlP**	$(T)_{24}$	F: TTAGACAGAAAAAACGACTC R: GAGAACGATTGGGATGGAAA	55	328–336	KC344387
GCP18 (HEX)	*psbA-trnK*-UUU*	$(T)_{18}(A)_9$	F: CTGTCATTTCTTTCCTTTCG R: ATTTCCCCGTTATCTCCCCC	55	338–347	KC344386
GCP27 (TAMRA)	*psaL-ycf4**	$(A)_8(A)_9$	F: TGACCCGATAAGGTGTTGTT R: CGGCTTCCGAAGTAACTTTT	55	179–181	KC344391
GCP4 (FAM)	*petN-psbM*	$(T)_{10}$	F: ACGGAGTATTACATAGGTTC R: AATACTGCTCTTTATGCTCC	55	225	KC344392
GCP9 (TAMRA)	*trnE*-UUC-*trnT*-GGU	$(T)_{10}$	F: ACCCGTAGCGATATTTGTTC R: TTTGTAGTATTCAGCACCCA	53	184	KC861980
GCP1 (FAM)	*cemA-petA*	$(TC)_8$	F: TATTCCTTTCCCTCTTTCTC R: TTACCTGTCCACGATTCTTC	51	108	KC861981
GCP2 (HEX)	*psbH-petB*	$(A)_{10}$	F: ATTCCCGTGAGTTGGGTCTA R: GATTTTAGCAAGTCGCACAC	55	193	KC861982
GCP3 (TAMRA)	*trnN*-GUU-*chlL*	$(TA)_6$	F: GGAAGGAGAGAGGAGGAATG R: GAAAGGGTTGAATGGTAG	53	233	KC861983
GCP7 (FAM)	*rps18-rpl20*	$(A)_9$	F: CAACAACGCCTAATGACTAT R: TAATGAAATAGGTGGAGCAG	50	290	KC861984
GCP8 (HEX)	*trnE*-UUC-*trnT*-GGU	$(TA)_7$	F: TTAGTCCTGAAACGATGAAC R: GATTTGAACCGATGACTTAC	50	199	KC861985
GCP15 (HEX)	*rsp7-trnL*-CAA	$(T)_9$	F: CCCACTCGGACTAAGACATA R: TAGTTGTTTCTGACCTCGCT	52	93	KC861986
GCP17 (FAM)	*trn*H-GUG-*psb*A	$(A)_{23}$	F: AGCAACACTGGCAGATAAAG R: GTAACGCTCACAACTTCCCT	53	211	KC861987
GCP19 (TAMRA)	*trnG*-GCC-*trnR*-UCU	$(T)_{28}$	F: CGCTACCCGCTCAGATTCCT R: TGGACGCTTTTTCTCCTTCT	57	233	KC861988
GCP21 (TAMRA)	*petB-petD*	$(T)_{25}$	F: AGGTATTCCAGGTCCTTTAT R: CTCCCATAATCCATCTTCTC	50	234	KC861989
GCP25 (FAM)	*trnT*-GGU-*psbD*	$(TA)_7$	F: CGTTATGATCTCTTCTCCTC R: GTGCTTTTCCCCTATTCGTA	51	254	KC861990
GCP26 (HEX)	*psal-ycf4*	$(AT)_9(AT)_7$	F: GGACACGAACAAAATAGAGA R: GTCAGTGTGATGGAAATACC	50	203	KC861991

Note: T_a = annealing temperature.
[a]The fluorescent dye label used for each forward primer is given in parentheses.
[b]Polymorphic loci are marked with an asterisk (*); all other loci in Table 1 are monomorphic.

produced amplicons matching the expected sizes. Polymorphisms of these 21 cpSSRs were assessed using 39 accessions from four populations, i.e., Jinfo Mountain, Chongqing Municipality (JF); Wuchuan, Guizhou Province (WC); Enshi, Hubei Province (ES); and Tianmu Mountain, Zhejiang Province (TM) (Appendix 1). As our previous studies (Gong et al., 2008a, 2008b) suggested, these populations are located in the two refugia. We repeated the experiments to verify the reproducibility of these amplicons. The forward primer of each pair was labeled with a fluorescent dye (6-FAM, HEX, or TAMRA). Amplification followed the conditions described above. Equal volumes of 10× diluted PCR products with three different dye-labeled primers were mixed with GeneScan 500 ROX Size Standard (Applied Biosystems, Carlsbad, California, USA). Fragment analyses were performed on a MegaBACE 1000 DNA Analysis System (GE Healthcare Biosciences, Pittsburgh, Pennsylvania, USA), and alleles were manually scored using GeneMarker version 1.97 (SoftGenetics, State College, Pennsylvania, USA). The resulting genotype data were analyzed using GenAlEx version 6.4.1 (Peakall and Smouse, 2006) to estimate number of alleles per locus (A) and unbiased haploid diversity (h).

Eight out of the 21 stably amplified cpSSR markers were polymorphic in the four analyzed populations of *G. biloba* (Table 1). At the species level, the number of alleles per locus ranges from three to seven, and the unbiased haploid diversity per locus varies from 0.441 to 0.807 (Table 2). At the population level, the values of these two parameters range from one to five and from 0.250 to 0.810, respectively (Table 2). Population TM exhibits the highest level of genetic diversity (A = 3.9, h = 0.627), exceeding populations WC (2.1 and 0.384) and JF (2.0 and 0.295), while population ES shows the lowest values (1.8 and 0.277).

CONCLUSIONS

The first 21 chloroplast microsatellite markers were developed for *G. biloba*, including 13 monomorphic and eight polymorphic ones. The alleles of the eight polymorphic chloroplast loci were verified to be reliable. This set of novel polymorphic and monomorphic cpSSR markers provides a valuable tool for population genetic and phylogeographic investigations of *G. biloba*. They can also be useful for plant breeding companies, seed/sapling testing agencies, and the wider scientific community in identifying maternal parents and distinguishing cultivars of ginkgo. Furthermore, given the conservative nature of cpDNA loci, these markers might also be useful for other gymnospermous

Table 2. Characteristics of eight polymorphic chloroplast microsatellite loci in four populations of *Ginkgo biloba*.[a]

	JF (n = 8)		WC (n = 8)		ES (n = 8)		TM (n = 15)		All (n = 39)	
Locus	*A*	*h*	*A*	*h*	*A*	*h*	*A*	*h*	*A*	*h*
GCP5	2	0.429	2	0.250	2	0.250	3	0.590	4	0.441
GCP6	2	0.250	2	0.429	1	0.000	4	0.733	4	0.656
GCP22	3	0.679	3	0.607	2	0.536	5	0.790	6	0.807
GCP23	2	0.250	2	0.250	1	0.000	5	0.705	7	0.640
GCP24	2	0.250	2	0.429	1	0.000	2	0.248	3	0.521
GCP20	1	0.000	2	0.429	2	0.250	4	0.695	6	0.771
GCP18	2	0.250	2	0.250	2	0.429	5	0.810	6	0.722
GCP27	2	0.250	2	0.429	3	0.750	3	0.448	3	0.582
Mean	2.0	0.295	2.1	0.384	1.8	0.277	3.9	0.627	4.9	0.642

Note: *A* = number of alleles per locus; *h* = unbiased haploid diversity; *n* = sample size.

[a]Locality information for the populations: JF = Jinfo Mountain, Chongqing Municipality; WC = Wuchuan, Guizhou Province; ES = Enshi, Hubei Province; TM = Tianmu Mountain, Zhejiang Province.

species, because three polymorphic cpDNA loci (i.e., *trnK* intron, *trnS-trnG* spacer [Gong et al., 2008a], and *atpH-atpI* spacer [Zhao et al., in preparation]) were successfully amplified in ginkgo using the universal markers.

LITERATURE CITED

Clarke, K. R., and R. N. Gorley. 2001. Primer v5: User manual/tutorial. Primer-E Ltd., Plymouth, United Kingdom.

Doyle, J. J., and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

Drummond, A. J., B. Ashton, M. Cheung, J. Heled, M. Kearse, R. More, S. Stones-Havas, and A. Wilson. 2008. Geneious version 4.5.5 created by Biomatters. Website http://www.geneious.com [accessed 8 December 2012].

Gong, W., C. Chen, C. Dobeš, C. X. Fu, and M. A. Koch. 2008a. Phylogeography of a living fossil: Pleistocene glaciations forced *Ginkgo biloba* L. (Ginkgoaceae) into two refuge areas in China with limited subsequent postglacial expansion. *Molecular Phylogenetics and Evolution* 48: 1094–1105.

Gong, W., Z. Zeng, Y. Y. Chen, C. Chen, Y. X. Qiu, and C. X. Fu. 2008b. Glacial refugia of *Ginkgo biloba* and human impact on its genetic diversity: Evidence from chloroplast DNA. *Journal of Integrative Plant Biology* 50: 368–374.

Kwant, C. 2013. The Ginkgo pages [online]. Website http://kwanten.home.xs4all.nl/ [accessed 28 June 2013].

Li, Q., and J.-M. Wan. 2005. SSRHunter: Development of a local searching software for SSR sites. *Hereditas* 27: 808–810. Website http://www.bio-soft.net/dna/SSRHunter.htm [accessed 3 July 2013].

Peakall, R., and P. E. Smouse. 2006. GenAlEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288–295.

Tang, C. Q., Y. C. Yang, M. Ohsawa, S. R. Yi, A. Momohara, W. H. Su, H. C. Wang, et al. 2012. Evidence for the persistence of wild *Ginkgo biloba* (Ginkgoaceae) populations in the Dalou Mountains, southwestern China. *American Journal of Botany* 99: 1408–1414.

Yan, X. F., C. L. Lian, and T. Hogetsu. 2006. Development of microsatellite markers in ginkgo (*Ginkgo biloba* L.). *Molecular Ecology Notes* 6: 301–302.

Yan, X. L., Y. Y. Chen, and C. X. Fu. 2009. Eleven novel microsatellite markers developed from the living fossil *Ginkgo biloba* (Ginkgoaceae). *Conversation Genetics* 10: 1277–1279.

Zhao, Y. P., J. Paule, C. X. Fu, and M. A. Koch. 2010. Out of China: Distribution history of *Ginkgo biloba* L. *Taxon* 59: 495–504.

Zhou, Z. Y. 2009. An overview of fossil Ginkgoales. *Palaeoworld* 18: 1–22.

Appendix 1. Population localities of the samples of *Ginkgo biloba* used in this study. All vouchers are deposited in the Herbarium of Zhejiang University (HZU), Zhejiang, China.

Population code	Population locality	Voucher	Geographic coordinates	Altitude (m)
ES	Yesanguan, Enshi, Hubei	W. Gong, 2004ES173	30°36′37″N, 110°18′29″E	675–1122
JF	Mt. Jinfo, Chongqing	C. X. Fu, 2003JF135	107°10′03″N, 29°01′03″E	800–1300
WC	Wuchuan, Guizhou	Y. Q. Ge, 2001WC011	108°06′49″N, 28°42′56″E	550–1200
TM	Mt. Tianmu, Zhejiang	C. Chuan, 2006TM301	119°26′15″N, 30°19′51″E	300–1100